PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:

Kocken et al.

Serial No.: 10/615,615

Filed: July 8, 2003

For: EFFICIENT EXPRESSION OF PLASMODIUM APICAL MEMBRANE ANTIGEN 1 IN YEAST CELLS

Confirmation No.: 8276

Examiner: R. Akhavan

Group Art Unit: 1636

Attorney Docket No.: 2183-6041US

CERTIFICATE OF MAILING

I hereby certify that this correspondence along with any attachments referred to or identified as being attached or enclosed is being deposited with the United States Postal Service as First Class Mail on the date of deposit shown below with sufficient postage and in an envelope addressed to the Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.

March 15, 2005
Date

Signature

Shirley Dougherty
Name (Type/Print)

COMMUNICATION

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

Enclosed is a certified copy of Priority Document 00204697.7 EP filed December 22, 2000 for the above referenced application.

Respectfully submitted,

Allen C. Turner
Registration No. 33,041
Attorney for Applicant(s)
TRASKBRITT
P.O. Box 2550
Salt Lake City, Utah 84110-2550
Telephone: 801-532-1922

Date: March 15, 2005
ACT/sdd

Europäisches Patentamt	European Patent Office	Office européen des brevets

Bescheinigung | Certificate | Attestation

Die angehefteten Unterlagen stimmen mit der ursprünglich eingereichten Fassung der auf dem nächsten Blatt bezeichneten europäischen Patentanmeldung überein.

The attached documents are exact copies of the European patent application described on the following page, as originally filed.

Les documents fixés à cette attestation sont conformes à la version initialement déposée de la demande de brevet européen spécifiée à la page suivante.

Patentanmeldung Nr. | Patent application No. | Demande de brevet n°

00204697.7

CERTIFIED COPY OF PRIORITY DOCUMENT

Der Präsident des Europäischen Patentamts;
Im Auftrag

For the President of the European Patent Office

Le Président de l'Office européen des brevets
p.o.

R C van Dijk

DEN HAAG, DEN
THE HAGUE, 22/02/05
LA HAYE, LE

Anmeldung Nr:
Application no.: 00204697.7
Demande no:

Anmeldetag:
Date of filing: 22.12.00
Date de dépôt:

Anmelder/Applicant(s)/Demandeur(s):

Stichting Biomedical Primate Research Center
Lange Kleiweg 151
2288 GJ Rijswijk
PAYS-BAS

Bezeichnung der Erfindung/Title of the invention/Titre de l'invention:
(Falls die Bezeichnung der Erfindung nicht angegeben ist, siehe Beschreibung.
If no title is shown please refer to the description.
Si aucun titre n'est indiqué se referer à la description.)

Efficient expression of plasmodium apical membrane antigen 1 in yeast cells

In Anspruch genommene Priorität(en) / Priority(ies) claimed /Priorité(s) revendiquée(s)
Staat/Tag/Aktenzeichen/State/Date/File no./Pays/Date/Numéro de dépôt:

/00.00.00/

Internationale Patentklassifikation/International Patent Classification/
Classification internationale des brevets:

C12N15/00

Am Anmeldetag benannte Vertragstaaten/Contracting states designated at date of filing/Etats contractants désignées lors du dépôt:

AT BE CH CY DE DK ES FI FR GB GR IE IT LI LU MC NL PT SE TR

Title: Efficient expression of *Plasmodium* apical membrane antigen 1 in yeast cells.

The invention relates to the fields of medicine, vaccines and diagnostics. More in particular the invention relates to the intervention with and the diagnosis of *Plasmodium* induced malaria.

Malaria is a wide-spread disease in most (sub)tropical countries. It is acquired by infection with a malaria parasite. The socioeconomic impact of this disease is enormous. Malaria exists in different forms, caused by different parasites. The symptoms vary considerably between the different forms. *Plasmodium vivax* and *Plasmodium falciparum* are the two most important human malaria parasites. Other human malaria parasites are *Plasmodium ovale* and *Plasmodium malariae*, but these two species are less pathogenic than *P. vivax* and *P. falciparum*. *P. vivax* causes less mortality than *P. falciparum*. Treatment of *P. falciparum* is becoming more complicated, because chloroquine resistant *P. falciparum* parasites are spreading rapidly and multi-drug resistant parasites have also developed. In addition, chloroquine resistant *P. vivax* has been detected, indicating similar problems in treatment of *P. vivax* as for *P. falciparum*.

At present, there is essentially no effective vaccine available against malaria, at least not for use in humans. Accumulated data, including that from non-human primate [1] [2] and rodent studies, [3] [4] have indicated that the apical membrane antigen-1 (AMA-1) family of molecules is a target for protective immune responses. In all *Plasmodium* species reported to date, with the exception of *Plasmodium falciparum* [5] and *P. reichenowi* [6] that form a phylogenetic clade distinct from other malaria parasites, AMA-1 is synthesized *de novo* as a 66 kDa transmembrane protein. The protein contains a predicted N-terminal signal sequence, an ectodomain, a predicted transmembrane region and a C-terminal cytoplasmic domain. The ectodomain is further divided into

three domains defined by disulfide bonds [7]. In *P. falciparum* and *P. reichenowi* the protein is expressed as an 83 kDa protein having an N-terminal extension as compared to the 66 kDa forms, referred to as the prosequence. Intra-species sequence polymorphism due to point mutations [8] [9] [10] reveals clustering of mutations in particular domains of the molecule. Despite this, between species there is considerable conservation of primary amino acid structure and predicted secondary structure. Evidence to date indicates that protection invoked by AMA-1 is directed at conformational epitopes [1] [3] [4] [11] located in the AMA-1 ectodomain. Immunisation with reduced AMA-1 fails to induce parasite inhibitory antibodies [3] [11] and so far only those monoclonal antibodies that recognize reduction-sensitive conformational AMA-1 epitopes have been shown to inhibit parasite multiplication *in vitro* for *P. knowlesi* [12] [13] and *P. falciparum* [6] [14]. This indicates that for an AMA-1 vaccine the correct conformation will be critical.

Recombinant expression of *P. falciparum* AMA-1 (Pf AMA-1) in a conformational relevant way that allows production of clinical grade material has been notoriously difficult. One characteristic important for recombinant expression techniques is the unusually high A+T content of *P. falciparum* codons in comparison to most other organisms and in particular in comparison to most other organisms generally used for recombinant protein expression. The group of Prof. Anders (WEHI, Australia) has developed expression of the ectodomain in *E. coli*, followed by a refolding protocol, but scaling up this process to levels that allow production of clinical grade material has proven cumbersome. Because eukaryotic expression systems are likely to produce material with the correct disulphide bonds directly, we have focused upon expression in such systems. Expression of the full length 622 amino acids long Pf AMA-1 protein (7G8 strain) in insect cells using recombinant baculovirus resulted in expression on the surface of insect cells [15]. The protein migrated in SDS-PAGE more slowly than the native molecule indicating glycosylation. Expression in the presence of tunicamycin confirmed this. Said Pf AMA-1

protein was used to raise rat monoclonal antibodies (mAbs), some of which could block parasite multiplication in an *in vitro* assay. These functional mAbs recognised a conformational epitope located in the ectodomain of Pf AMA-1. Reactivity with these mAbs, especially with mAb 4G2, is used as one assay for proper folding of recombinant Pf AMA-1. Relatively low expression levels did not allow the baculovirus sytem to be developed for the production of clinical grade material.

We have obtained high level expression of *P. vivax* AMA-1 (Pv AMA-1) ectodomain in the methylotrophic yeast *Pichia pastoris* [16]. However, this expression system is not likewise suitable to produce a secreted ectodomain of Pf AMA-1. Using the same expression vector as has successfully been used for Pv AMA-1, recombinant Pf AMA-1 *P. pastoris* clones do not express Pf AMA-1 ectodomain at any level. Analysis of total RNA extracted from induced cultures revealed only truncated mRNA products for Pf AMA-1. So no effective expression of Pf AMA-1 was possible until the present invention. This was a problem because expression of homogeneous Pf AMA-1 in high amounts is highly desirable. Efficient production of Pf AMA-1 gives possibilities to develop a diagnostics, or a vaccine and/or a medicine against *P. falciparum* and/or other *Plasmodium* species. Presently, such a vaccine or medicine is not available.

The present invention provides a method for producing mRNA encoding *Plasmodium* AMA-1 ectodomain, or a functional part, derivative and/or analogue thereof, in a yeast cell, comprising providing said yeast cell with a nucleic acid encoding said *Plasmodium* AMA-1 ectodomain or functional part, derivative and/or analogue thereof, said nucleic acid being modified to utilize said yeast's codon usage. Preferably, said ectodomain is derived from a 83 kDa AMA-1 protein. Particularly the ectodomain of 83 kDa AMA-1 proteins are difficult to express in yeast cells. More preferably, said 83 kDa AMA-1 protein is derived from *Plasmodium falciparum*. Now that a method of the invention is

available, it is also possible to produce an analogous protein, such as a complete AMA-1 protein. Thus the invention also provides a method for producing mRNA encoding *Plasmodium* AMA-1 protein, or a functional part, derivative and/or analogue thereof, in a yeast cell, comprising providing said yeast cell with a nucleic acid encoding said *Plasmodium* AMA-1 protein, said nucleic acid being modified to utilize said yeast's codon usage. AMA-1 ectodomain produced with a method of the invention comprises at least one conformational epitope that is comparable to a conformational epitope in native AMA-1 ectodomain, produced by the parasite, preferably in a human host. AMA-1 ectodomain of the invention can be used for vaccination purposes and for diagnostic purposes.

With a method of the invention, it is possible to obtain mRNA encoding AMA-1 ectodomain in a yeast cell. In said yeast cell, said mRNA is efficiently translated into a functional AMA-1 ectodomain. With the teachings of the invention, a person skilled in the art is able to produce a functional part, derivative and/or analogue of said ectodomain comprising at least one immunogenic property of native ectodomain in kind not necessarily in amount.

In a preferred embodiment a method of the invention further comprises allowing for expression of said *Plasmodium* AMA-1 ectodomain or functional part, derivative and/or analogue thereof in said yeast cell. Preferably, said AMA-1 ectodomain or functional part, derivative and/or analogue thereof is purified from said yeast cell and/or culture medium.

A functional part of a *Plasmodium* AMA-1 ectodomain is defined as a part which comprises at least one immunogenic property of said AMA-1 ectodomain in kind, not necessarily in amount. In one embodiment said part comprises a subdomain of ectodomain, which can be defined for instance by disulphide bond patterning [7]. By immunogenic property is meant the capability to induce an immune response in a host. Preferably, said immunogenic property comprises a property to induce an immune response against a conformational epitope on a native AMA-1 ectodomain. A functional

derivative of a *Plasmodium* AMA-1 ectodomain is defined as a *Plasmodium* AMA-1 ectodomain which has been altered such that at least one immunogenic property of said molecule is essentially the same in kind, not necessarily in amount. A derivative can be provided in many ways, for instance through conservative amino acid substitution. A derivative can also be a fusion of AMA-1 ectodomain or a part thereof with a second protein. In a preferred embodiment said derivative comprises one or more amino acids from variant AMA-1 ectodomains. The resultant AMA-1 ectodomain is a consensus AMA-1 ectodomain having no naturally occurring counterpart. A person skilled in the art is well able to generate analogous compounds of a *Plasmodium* AMA-1 ectodomain. This can for instance be done through screening of a peptide library. Such an analogue comprises at least one immunogenic property of a *Plasmodium* AMA-1 ectodomain in kind, not necessarily in amount. For the present invention complete AMA-1 protein and shorter versions comprising a complete ectodomain are analogous to ectodomain.

Compared to the reported Pf AMA-1 genes, the A+T(U) content of a nucleic acid of the invention is reduced without changing amino acid sequences (with the exception of glycosylation sites, as described below). Preferably said A+T(U) content is reduced in a putative yeast polyadenylation consensus sequence to prevent premature termination of transcription. Such sequences are highly A+T rich and are thus more likely to be present within the A+T rich coding sequences of *P. falciparum* genes. Thus, one embodiment of the invention discloses a method of the invention, wherein at least one putative yeast polyadenylation consensus sequence in said nucleic acid has been modified.

Another problem for expression in eukaryotic systems is N-glycosylation. *P. falciparum* blood stage proteins are not N-glycosylated by the parasite. However, Pf AMA-1 contains 6 N-glycosylation sites that are

potentially recognised by other eukaryotic systems. Full length 7G8 Pf AMA-1 expressed in insect cells is glycosylated. Expression of Pv AMA-1 ectodomain in *Pichia* showed heterogeneous glycosylation of the recombinant product [16]. This could only partly be prevented by the addition of extremely high levels of tunicamycin to induction cultures, at the cost of a large drop in expression levels. Deglycosylation using N-glycosidase F was only complete after full denaturation of the protein, a process which would need refolding protocols to obtain properly folded material. Therefore, a preferred embodiment of the invention discloses a method of the invention, wherein at least one site in said *Plasmodium* AMA-1 ectodomain or functional part, derivative and/or analogue thereof that is generally glycosylated by eukaryotic expression systems, is removed. Said sites may generally be glycosylated by eukaryotic expression systems through the N-glycosylation pathway. Said site may be removed by mutating the nucleic acid sequence encoding said site. This may lead to a change of at least one amino acid composing said site. Said change may decrease said eukaryotic system's capability of glycosylating said site. Alternatively, amino acids which are part of said site may be removed without substitution. This may be accomplished by removing a part of the nucleic acid encoding said site.

The reasoning for removing a glycosylation site is three-fold. Firstly the presence and location of N-linked glycosylation can have profound but unpredictable targeting and focusing effects on the immune response to proteins [17]. In this context, the Pf AMA-1 baculovirus product had been used in protection studies in Aotus monkeys. These unpublished studies did not show a protective effect of AMA-1 immunisation. Although one explanation for this may have been that a sub-optimal adjuvant was used to formulate the antigen, we reasoned that the glycosylation of the Pf AMA-1 may also have significantly influenced the immune response in a non-beneficial way. Secondly glycosylation is frequently heterogeneous (as demonstrated by

expression of the native sequence Pv AMA-1 ectodomain in Pichia). Heterogeneous products may be difficult to reproducibly purify to acceptable standards under GMP, and such heterogeneity may create batch to batch variation in an immunogenic property of the product (given the published effects of N-linked glycosylation on immunogenicity). Thirdly, we wished to produce a protein with the least heterogeneity in order to prepare crystals for crystallographic determination of structure. It is generally accepted that the more homogeneous the protein, the higher the chances of successful crystal formation.

Based on the molecular weight of expressed AMA-1 protein in various *Plasmodium* species two groups of *Plasmodium* species can be identified. Those expressing an AMA-1 protein of approximately 66 kDa and those expressing an AMA-1 protein of approximately 83 kDa. A method of the invention is particularly suited to increase levels of expression of ectodomain of the approximately 83 kDa AMA-1 protein in yeast. Measuring the exact molecular weight of a protein is always a difficult task, thus for the present invention the number of 83 kDa should be taken as a guidance for the actual molecular weight of said AMA-1 protein. Variations of 10% in the estimates for molecular weight of a given protein are not abnormal. However, considering the large difference between the two variants of AMA-1 (66 versus 83 kDa) the size indication is only required to help a person skilled in the art determine whether the AMA-1 protein at hand belongs to one or the other class. A variation in the molecular weight measurements of 10% can easily be tolerated while still being able to select one of the two classes of AMA-1 proteins. Thus in a preferred embodiment of the invention said *Plasmodium* belongs to the clade whose members normally express said AMA-1 protein as an approximately 83 kDa protein. "Normally" is herein defined as under conditions occurring in nature. As has already been described in this disclosure, *P. falciparum* and *P. reichenowi* belong to said clade which has the

characteristic of expressing said AMA-1 protein as an 83 kDa protein. Another preferred embodiment of the invention discloses a method of the invention, wherein said *Plasmodium* comprises *Plasmodium falciparum*. Preferably, said *Plasmodium* comprises *Plasmodium falciparum* FVO. We have developed the Pf AMA-1 sequence from the FVO strain of *P. falciparum* for expression in *P. pastoris* for several reasons. The challenge strain that is likely to be used in phase II clinical trials is the 3D7 clone of NF54. The FVO strain has an AMA-1 sequence that is one of the most divergent from 3D7 reported to date, and therefore immunisation with FVO AMA-1 would allow for a markedly heterologous challenge. Because of the possibility that polymorphism in AMA-1 is selected and maintained because of immune pressure, the availability of two extremes of diversity for clinical testing apart and in combination will be extremely informative. In addition, the FVO strain has been adapted to grow in *Aotus lemurinus griseimembra* monkeys, thus allowing preclinical evaluation with homologous challenge possibilities in this non human primate system. FVO as well as 3D7 strains react with mAb 4G2, showing epitope conservation between the divergent AMA-1 sequences.

Another preferred embodiment of the invention discloses a method according to the invention, wherein said yeast is *Pichia*. Yet another preferred embodiment of the invention discloses a method according to the invention, wherein said yeast is *Pichia pastoris*.

In another aspect the present invention discloses an isolated and/or recombinant nucleic acid sequence encoding *Plasmodium* AMA-1 ectodomain or a functional part, derivative and/or analogue thereof, said nucleic acid being modified to utilize a yeast's codon usage. A functional part, derivative and/or analogue of an AMA-1 ectodomain comprises at least one conformational epitope of native AMA-1 ectodomain, preferably said conformational epitope is an ectodomain epitope.

As has been described above, preferably at least one putative yeast polyadenylation consensus sequence has been modified in a nucleic acid of the invention. Also, preferably at least one site in said *Plasmodium* AMA-1 ectodomain, or functional part, derivative and/or analogue thereof, that is generally glycosylated by eukaryotic expression systems, is removed. Thus, in a preferred aspect the invention discloses an isolated and/or recombinant nucleic acid sequence according to the invention, wherein at least one putative yeast polyadenylation consensus sequence has been modified. In another preferred aspect the invention discloses an isolated and/or recombinant nucleic acid sequence according to the invention wherein at least one site in said ectodomain or functional part, derivative and/or analogue thereof that is generally glycosylated by eukaryotic expression systems, is removed.

Figure 1 shows a nucleic acid of the invention, comprising above mentioned preferred characteristics. Thus, in one aspect the present invention discloses an isolated and/or recombinant nucleic acid sequence encoding *Plasmodium* AMA-1 ectodomain or a functional part, derivative and/or analogue thereof, said nucleic acid comprising a sequence as depicted in figure 1.

Considering that in the present invention a nucleic acid sequence was generated that can be used to express high amounts of *Plasmodium* AMA-1 ectodomain in a yeast cell and the fact that AMA-1 amino acid sequences of various species comprise significant homology, the present invention further provides a nucleic acid sequence encoding *Plasmodium* AMA-1 ectodomain or a functional part, derivative and/or analogue thereof, capable of hybridising to at least a functional part of a nucleic acid of the invention. Through said hybridisation criterion, it is warranted that said nucleic acid sequence comprises similar expression characteristics (in kind not necessarily in amount) in yeast cells, at least on mRNA level as the nucleic acid of figure 1. By at least a functional part of a nucleic acid of the invention is meant a part

of said nucleic acid, at least 30 base pairs long, preferably at least 200 base pairs long, comprising at least one expression characteristic (in kind not necessarily in amount) as a nucleic acid of the invention. Preferably but not necessarily said part comprises an immunogenic property of an AMA-1 ectodomain. In one aspect of the invention said hybridising to at least a functional part of a nucleic acid of the invention is under stringent conditions.

In another aspect, the invention provides an AMA-1 specific nucleic acid sequence comprising at least 50 percent homology to a nucleic acid sequence of the invention. An AMA-1 specific nucleic acid sequence is defined herein as a nucleic acid sequence, comprising at least 20 nucleotides, preferably at least 50 nucleotides, said sequence comprising a nucleic acid sequence corresponding to at least part of an AMA-1 gene, or comprising a nucleic acid sequence which is complementary to a sequence corresponding to at least part of an AMA-1 gene.

In a preferred aspect of the present invention, said AMA-1 specific nucleic acid sequence comprises at least 60 percent homology to a nucleic acid of the invention. More preferably, said AMA-1 specific nucleic acid sequence comprises at least 75 percent homology to a nucleic acid of the invention. In a most preferred aspect of the invention, said AMA-1 specific nucleic acid sequence comprises at least 90 percent homology to a nucleic acid of the invention.

With the teachings of the present invention, a person skilled in the art is capable of generating a nucleic acid sequence comprising an immunogenic property of an AMA-1 ectodomain from another species of *Plasmodium*, for instance *Plasmodium vivax* while still using essentially the same nucleic acid sequence as given in figure 1. Such variant nucleic acid will of course still be capable to hybridise to at least a functional part of the nucleic acid depicted in figure 1.

In a preferred embodiment the present invention discloses a nucleic acid sequence according to the invention, wherein said *Plasmodium* belongs to the

clade whose members express said AMA-1 protein as an approximately 83 kDa protein. As has been described before, *P. falciparum* and *P. reichenowi* belong to said clade. More preferably, said *Plasmodium* comprises *Plasmodium falciparum*. More preferably, said Plasmodium comprises *Plasmodium falciparum* FVO.

A nucleic acid of the invention may, for instance, encode a derivative of a *Plasmodium* AMA-1 ectodomain or part thereof, comprising one or more amino acids from variant AMA-1 ectodomains. The resultant AMA-1 ectodomain or part thereof is a consensus AMA-1 ectodomain having no naturally occurring counterpart. Thus, in one aspect the invention provides a nucleic acid sequence according to the invention, wherein said *Plasmodium* AMA-1 ectodomain or functional part, derivative and/or analogue thereof comprises a consensus *Plasmodium* AMA-1 ectodomain or functional part, derivative and/or analogue thereof. In a preferred embodiment said part of an AMA-1 ectodomain comprises at least one immunogenic property of said ectodomain. In another aspect, a nucleic acid of the invention may be modified to utilize codon usage of *Pichia*. Thus, in one aspect the invention provides a nucleic acid sequence according to the invention, wherein said yeast is *Pichia*. Preferably, said yeast is *Pichia pastoris*.

A nucleic acid of the invention is particularly suitable for efficient expression of *Plasmodium* AMA-1 ectodomain or a functional part, derivative and/or analogue thereof. Therefore, in another aspect the present invention provides a method for producing *Plasmodium* AMA-1 ectodomain or a functional part, derivative and/or analogue thereof, comprising:
-providing a yeast cell with a nucleic acid according to the invention, and
-collecting formed *Plasmodium* AMA-1 ectodomain or functional part, derivative and/or analogue thereof.

Preferably, said yeast is *Pichia* yeast, more preferably *P. pastoris*. Alternatively, it is possible to express a nucleic acid of the invention in another eukaryotic system, for instance baculovirus or a CHO cell. It is even possible to express a nucleic acid of the invention in bacteria. Said eukaryotic systems, and bacteria, are more capable of expressing a nucleic acid utilizing yeast's codon usage, compared to a nucleic acid utilizing *P. falciparum*'s codon usage. However, a nucleic acid of the invention can also be modified to utilize codon usage of said other eukaryotic systems, or bacteria. Preferably, said nucleic acid has been modified to remove at least one putative polyadenylation consensus sequence which is recognised by said other eukaryotic system. More preferably, at least one site in said nucleic acid that is generally glycosylated by said other eukaryotic expression system, is removed. Expression of a nucleic acid of the invention in another eukaryotic system, or bacteria, as mentioned above, is still within the scope of the present invention.

Of course, by using a method as previously described, *Plasmodium* AMA-1 ectodomain or a functional part, derivative and/or analogue will be produced. Thus, another aspect of the invention provides a *Plasmodium* AMA-1 ectodomain or a functional part, derivative and/or analogue thereof, obtainable by a method of the invention. The invention further provides a Pf AMA-1 ectodomain or functional part, derivative and/or analogue thereof, produced in a yeast cell. In a preferred embodiment said AMA-1 ectodomain or functional part, derivative and/or analogue thereof is purified. As is described in example 4.3, with a method of the invention it is possible to obtain the desired Pf AMA-1 ectodomain, without contaminants like for instance a 50 kDa contaminant. Thus, in a preferred aspect, the invention provides a method according to the invention, further comprising purifying said *Plasmodium* AMA-1 ectodomain or functional part, derivative and/or analogue thereof.

A cell producing said *Plasmodium* AMA-1 ectodomain, or a functional

part, derivative and/or analogue thereof, by a method as previously described is of course also within the scope of the present invention. So yet another aspect of the invention provides an isolated cell comprising a nucleic acid of the invention.

In yet another aspect the invention provides an isolated cell comprising a *Plasmodium* AMA-1 ectodomain of the invention or functional part, derivative and/or analogue thereof.

Plasmodium AMA-1 is particularly well suited for the preparation of a vaccine, because accumulated data have indicated that this family of molecules is a target for protective immune responses. As the present invention provides a way of producing a *Plasmodium* AMA-1 ectodomain or a functional part, derivative and/or analogue thereof efficiently, the invention also provides a use of a *Plasmodium* AMA-1 ectodomain or functional part, derivative and/or analogue thereof according to the invention for the preparation of a vaccine. Of course, this vaccine is particularly well suited for the prophylaxis of malaria. Thus, the invention provides a use of a *Plasmodium* AMA-1 ectodomain or functional part, derivative and/or analogue thereof according to the invention for the preparation of a vaccine for prevention of malaria.

In a particular embodiment, the invention provides a use of a *Plasmodium* AMA-1 ectodomain or functional part, derivative and/or analogue thereof according to the invention for the preparation of a vaccine for prevention of malaria, wherein said malaria is caused by *Plasmodium falciparum*.

A *Plasmodium* AMA-1 ectodomain according to the invention is also well suited for diagnosis of malaria. A person skilled in the art can think of many ways of determining the presence of *Plasmodium* AMA-1 ectodomain, or antibodies against *Plasmodium* AMA-1 ectodomain, in a patient. One way is

for instance collecting a blood sample of a patient. Said blood sample can be administered to a well which contains *Plasmodium* AMA-1 ectodomain of the invention. If the patient contains antibodies against *Plasmodium* AMA-1 ectodomain, they will bind to the *Plasmodium* AMA-1 ectodomain in the well. These antibodies can be made visible by many techniques known in the art, for instance by incubation with fluorescent labeled rabbit anti human antibodies. Many other ways are known in the art which are still within the scope of the present invention. Thus, the present invention provides a use of a *Plasmodium* AMA-1 ectodomain or functional part, derivative and/or analogue thereof according to the invention for diagnosis of malaria.

Another embodiment provides a method for, at least in part, diagnosis of malaria, comprising collecting a sample from an individual and providing *Plasmodium* AMA-1 ectodomain or functional part, derivative and/or analogue thereof of the invention with at least part of said sample. Preferably, said sample is a blood sample.

Another embodiment of the present invention discloses a method for, at least in part, prophylaxis of malaria, comprising administering a vaccine according to the invention to an individual. However, an immune response is often only high directly after administration of a vaccine to an individual. Therefore, a preferred embodiment of the present invention provides a method for, at least in part, prophylaxis of malaria comprising administering to an individual slow release compositions comprising said vaccine.

By slow release composition is meant a composition from which a vaccine of the invention is only slowly migrated into the body. This way, said body contains a vaccine of the invention for a prolonged period, so the immune-response will be high during a prolonged period of time.

The following examples illustrate the present invention. The examples do not limit the present invention in any way. A person skilled in the art can perform alternative ways which are still in the scope of the present invention.

EXAMPLES

1 Development of synthetic gene for *P. falciparum* FVO strain Pf AMA-1.

1.1 Original FVO sequence

Cryopreserved parasite stocks from *P. falciparum* FVO were prepared from an infected *Aotus lemurinus griseimembra* monkey at the young ring stage of development and DNA was isolated (Gentra systems Inc., Minneapolis, MN) directly from a parasite stock according to the manufacturer's instructions. Pf AMA-1 was amplified by polymerase chain reaction using *Pfu* polymerase (Promega, Leiden, The Netherlands) and primers PF83A: 5'-GGGGGATCCATGAGAAAATTATACTGCGTATT-3' (nt 1-23 and additional *Bam*HI restriction site) and PF83B: 5'-ACGTGGATCCTTAATAGTAT-GGTTTTTCCATCAGAACTGG-3' (complementary to nt 1843-1869 and additional *Bam*HI restriction site) containing *Bam*HI restriction sites to facilitate cloning in pBluescript. A pool of four independent clones was used for sequence analysis using an ABI Prism™ 310 automated sequencer (PE Applied Biosystems, Foster City, CA) according to the manufacturers instructions, and primers previously synthesised for sequencing of Pf AMA-1 [10]. This resulted in the unambiguous sequence of *P. falciparum* FVO Pf AMA-1, that differs from the FVO AMA-1 sequence available from Genbank (accesssion number U84348) at three amino acid positions. The most notable difference is that the Genbank FVO AMA-1 sequence is one amino acid shorter than any other available AMA-1 sequence, and our FVO AMA-1 sequence does not have this deletion.

1.2 Alteration of N-glycosylation sites

The sequence of gene Pf AMA-1 from FVO strain that we have established encodes a protein of 622 amino acid residues that has 6 potential N-glycosylation sites. Our previous experience with expressing Pf AMA-1 in

baculovirus/insect cells as well as with expressing Pv AMA-1 in *P. pastoris* has shown that these N-glycosylation sites will be glycosylated in eukaryotic heterologous expression systems. As explained above, this is undesirable since native Pf AMA-1 is not glycosylated. Therefore we developed a variant that exploited the lack of conservation of N-glycosylation sites in published *Plasmodium* AMA-1 allele sequences. Asn 162 was changed to Lys that is present in that position in Thai-Tn strain Pf AMA-1 (accession nr M58547). Thr 288 was changed to Val (present in *P. vivax* and *P. knowlesi* AMA-1; accession nrs Y16950 and M61097); Ser 373 was changed to Asp (present in *P. knowlesi* AMA-1); Asn 422 and Ser 423 were changed to Asp and Lys, respectively (present in *P. knowlesi*, *P. vivax*, *P. chabaudi* (accession nr M25248) and *P. fragile* AMA-1(accession nr M29898)) and Asn 499 was changed to Glu (present in *P. chabaudi* AMA-1).

1.3 Synthetic gene with *P. pastoris* codon usage

The nucleotide sequence with the six changed codons to delete the potential N-glycosylation sites was used to develop a synthetic gene utilising the codon usage of *P. pastoris* (NIMR, London). Our previous experience with expressing Pf AMA-1 in *P. pastoris* taught us that the high A+T content of the *P. falciparum* gene makes it extremely difficult to express this in *P. pastoris*. There are several A+T rich regions within the coding sequence that are recognised as transcription termination and/or polyadenylation sites in yeast, resulting in truncated mRNAs and no protein production. The sequence of the synthetic gene was designed according to *P. pastoris* codon usage with the aid of the CODOP program [18]. This program allows codon optimisation with host organism preference. It enabled design of an optimal sequence, with strategic insertion of restriction sites, and the generation of oligos of 40 nucleotides in length from both strands of the gene. The resulting set of 92 oligos was rigorously screened for the presence of potential transcription termination signals and undesirable repeats, inverted repeats, and regions of

complementarity which could potentially lead to nonspecific intermolecular hybridisation. The 20 nucleotide overlap between each 40-mer primer was adjusted to give a melting temperature in the range 68-62 °C, in order to allow subsequent use of the primers for DNA sequencing. Gene synthesis was by assembly polymerase chain reaction (PCR), using the proof-reading *Pfu* DNA polymerase, as described in reference [18]. Blunt-ended PCR products corresponding to each 'half' of the gene were cloned into *pMos*Blue (Amersham Pharmacia) and fully sequenced on both strands before subcloning to produce the complete synthetic gene. The final product was again sequenced on both strands. The sequence of the synthetic gene FVO Pf83syn is provided in figure 1.

2 Expression of FVO Pf83syn ectodomain in *P. pastoris*

2.1 Development of expression constructs

For secreted expression in *P. pastoris* strain KM71H we used vector pPICZαA (Invitrogen). This vector provides an N-terminal signal sequence and a C-terminal myc epitope followed by a 6 x His tag for easy purification. Gene fragments have to be cloned in frame with these sequences. Primers for PCR amplification of the Pf AMA-1 ectodomain were Pf83A: 5'-GGAATTCCAGAACTACTGGGAGCATCC-3' (nt 73-92 and additional *Eco*RI restriction site) and Pf83H: 5'-GCTCTAGAATGTTATCGTAACGTAGGCTT-3' (complementary to nt 1615-1634 and additional *Xba*I restriction site) or Pf83A and Pf83I: 5'-GCTCTAGACTACATGTTATCGTACGTAGGCTT-3' (complementary to nt 1615-1635, plus stopcodon plus additional *Xba*I restriction site; this provides the full ectodomain without myc epitope and His tag). A 50 µL PCR reaction contained 10 ng template DNA (FVO Pf83syn), 100 ng of each of the primers Pf83A and Pf83H, or Pf83A and Pf83I, 0.2 mM dNTP, 5 µL 10x *Pfu* reaction buffer and 1 unit *Pfu* polymerase (Promega). Amplification proceeded as follows: 1 min, 94°C, 1 min 52°C, 1.5 min 72°C for 3

cycles; 1 min, 94°C, 1 min 60°C, 1.5 min 72°C for 30 cycles; 5 min, 72°C and then stored at 4°C. The resulting 1578 bp PCR product was digested with *Eco*RI and *Xba*I sequentially, and ligated into *Eco*RI/*Xba*I digested pPICZαA in a 1:10 molar ratio. *E. coli* DH5α subcloning efficiency cells were transformed with 5 µL of the ligation mixture and plated on low salt LB plates containing 25 µg/ml zeocin and cultured overnight at 37°C. Colonies were grown in low salt LB containing 25 µg/ml zeocin, plasmids were isolated by standard miniprep methods and analysed by restriction enzyme digestion. One clone containing the correct insertion for each of the PCR products (named Pf4mH for primers A and H, and Pf11-0 for primers A and I) was used to isolate plasmid DNA for transformation of *P. pastoris*.

2.2 *Pichia* transformation and analysis

The expression construct was linearised with *Sst*I and 10 µg DNA was used to transform 80 µL *P. pastoris* KM71H cells by electroporation following the Invitrogen protocols. 1 ml of 1M sorbitol was added and the cells were allowed to recover for 2h at 30°C. Cells were then plated (25, 50, 100, 200 µL aliquots) on YPDS (1% yeast extract, 2% peptone, 2% dextrose, 1 M sorbitol) agar plates containing 100 µg/ml zeocin, and incubated for 4 days at 30°C. Colonies were picked and grown for 2 days at 30°C in 10 ml of BMGY (1% yeast extract, 2% peptone, 1.34% Yeast Nitrogen Base, 1% glycerol, 0.4 mg/L biotin, 0.1M K-phosphate pH 6.0) in 50 ml Falcon tubes with vigorous shaking. Cells were harvested by low-speed centrifugation, resuspended in 4 ml of BMMY (BMGY with glycerol substituted for 0.5% methanol), and cultured for an additional 2 days. Cells were harvested and the culture supernatants were analysed for the presence of Pf AMA-1 ectodomain by SDS-PAGE. Gels were stained with Coomassie Brilliant Blue. All clones analysed expressed an equal amount of two proteins in the culture supernatant. A 50 kDa molecule of thus far unknown origin as well as an approximately 75 kDa protein, which proved to be the Pf AMA-1 ectodomain, with or without myc epitope and His tag

(Pf4mH and Pf11-0, respectively). Expression levels in these small scale cultures are estimated to be 50 mg/L. Our experience with the expression of Pv AMA-1 in *P. pastoris* suggests that this might result in levels approaching 1 g/L in optimised fermentations. No obvious degradation products were visible in the culture supernatants.

Culture supernatants of Pf4mH were spot blotted on nitrocellulose membranes and incubated with rat monoclonal antibody 58F8 (recognising a linear epitope in the N-terminal region of Pf AMA-1), or 4G2 (recognising a conformational epitope in the ectodomain and capable of blocking parasite multiplication *in vitro*) for 1 h at room temperature. After incubation with goat-anti-rat IgG, colour was developed using NBT/BCIP. Only culture supernatants from the recombinant *P. pastoris* expressing the 75 kDa protein reacted with both mAbs. Control culture supernatants, where the 50 kDa protein, but not the 75 kDa protein, was present did not react with either of the mAbs. This indicates that the 75 kDa protein is the Pf AMA-1 ectodomain and that the secreted material is properly folded. As expected, reactivity with 4G2 was lost when the culture supernatant was reduced with β-mercaptoethanol prior to spot blotting, demonstrating the correct disulfide bond formation within the ectodomain to recreate the 4G2 epitope.

Purified Pf4mH (sec 4) was used in a standard ELISA to test reactivity with mAb 4G2 and a human serum from an African endemic region. These human sera show high reactivity with conformational epitopes of AMA-1, and hardly react with reduced AMA-1. In this ELISA, strong reactivity with 4G2 and the human serum was detected, whereas a control mAb and a pool of European human serum did not react. As a positive control, similar amounts of baculovirus produced Pf AMA-1 were coated on an ELISA plate and incubated with the same serum samples. Similar results were obtained, although reactivity was much lower, suggesting a much better quality for the *Pichia* Pf4mH product.

3 Bulk production

For mid-scale production of Pf AMA-1 ectodomain recombinant *P. pastoris* was cultured in 1L baffled flasks (400 ml BMGY per flask) for 48 h at 29-30°C under vigorous shaking. Cells were harvested and resuspended in 100 ml BMMY, and then cultured for 48 h at 29-30°C under vigorous shaking. Methanol was added to a final concentration of 0.5% every 24 h. After low-speed centrifugation, the culture supernatant was harvested. Protein was precipitated with amonium sulphate (70% final concentration) at 0°C, and the precipitate was stored at 4°C until use.

4 Purification strategies

4.1 Purification of Pf4mH on Ni resins

Additional proof that the secreted 75 kDa protein is the Pf AMA-1 ectodomain comes from purification using Ni resins, since recombinant proteins produced using the pPICZα vector contain His tags that have a high affinity for Ni. The ammonium sulphate precipitate of 50 ml culture supernatant was solubilised in 2 ml binding buffer (20 mM Na Phosphate pH 7.8, 0.5 M NaCl) and loaded on an 8 ml Ni-agarose column (Probond, Bio-Rad) at 0.2 ml/min. The column was washed at 1 ml/min with 15 ml binding buffer, 25 ml of the same buffer pH 6.0, 15 ml of the buffer pH 5.5 and then eluted with the same buffer at pH 4.0. Elution was monitored at 280 nm. The pH 4.0 peak fractions contained a single protein of 75 kDa as determined by SDS-PAGE analysis. Alternatively, the 75 kDa ectodomain could be eluted with a linear 0-500 mM Imidazole gradient in 20 mM Na Phosphate pH 6.0, 0.5 M NaCl. Spot blotting of the peak fractions revealed strong 4G2 and 58F8 binding, indicating that the 75 kDa protein is the His-tagged Pf AMA-1 ectodomain. The 50 kDa protein present in the culture supernatant as well as yellow-stained flavin components were present in the flow through and pH 6.0 wash fractions.

4.2 Other purification strategies for Pf11-0

Other purification strategies are needed when the ectodomain is expressed without His tag, which might be more appropriate for clinical purposes. One way of purifying the 75 kDa ectodomain Pf11-0 away from the 50 kDa protein is the use of hydroxy apatite (HAP) [19] [20] chromatography.

The ammonium sulphate precipitate of 100 ml culture supernatant was solubilised in 5 ml 10 mM $NaPO_4$, pH 6.8 and loaded onto a prepacked 5 ml HAP column (CHT-II, Bio-Rad) at 0.5 ml/min. Elution with a 20 ml gradient to 400 mM $NaPO_4$, pH 6.8 at 1 ml/min was monitored at 280 nm. Two overlapping peaks were evident, the first one containing mainly the 50 kDa protein, the second one mainly the Pf AMA-1 ectodomain. Further purification could be obtained by subsequent anion exchange chromatography of the pooled second peak fractions after diluting 1:10 in miliQ water on a prepacked 5 ml UNO Q column (Bio-Rad), eluted with a linear gradient of 0-0.5 M NaCl in 20 mM Tris.HCl pH 7.6. This results in several peaks containing the remainder of the 50 kDa contaminant as well as several degradation products of the AMA-1 ectodomain, and a single peak that contains pure intact AMA-1 ectodomain, as analysed by reduced SDS-PAGE and Coomassie staining.

4.3 Production of Pf11-0 without the contaminating 50 kDa protein

The 50 kDa protein present in the culture supernatant of our recombinant *P. pastoris* KM71H clones is not common (information from Invitrogen). Transformation of just the empty pPICZα vector into the same batch of *P. pastoris* KM71H also yielded a 50 kDa protein in the culture supernatant upon methanol induction. Untransformed *P pastoris* KM71H does not produce this protein. We have now succeeded in preparing a new clone (Pf11-0.1) that only secretes the 75 kDa Pf AMA-1 ectodomain upon methanol induction, and that does not produce the 50 kDa contaminant. This was achieved by picking a single colony of *P. pastoris* KM71H from a freshly

prepared agar plate, made from the original stock of that strain. This colony was used to start fresh cultures, that were transformed with the Pf11-0 vector, resulting in the above described expression.

Purification as described under 4.2 will provide higher yields of pure Pf AMA-1 ectodomain, since there is no need to separate the 75 kDa product from a major contaminant any more, allowing to take the complete peak fraction from the HAP column for further anion exchange chromatography purification.

Brief description of the drawings

Figure 1: sequence of an isolated and/or recombinant nucleic acid of the invention, encoding *Plasmodium* AMA-1 ectodomain. Surprisingly, this sequence is very well expressed in *Pichia pastoris*, whereas a nucleic acid sequence encoding wild-type Pf AMA-1 ectodomain is not.

References

1. Collins, W.E., et al., Protective immunity induced in squirrel monkeys with recombinant apical membrane antigen-1 of Plasmodium fragile. Am J Trop Med Hyg, 1994. 51(6): p. 711-9.

2. Deans, J.A. and W.C. Jean, Structural studies on a putative protective Plasmodium knowlesi merozoite antigen. Mol Biochem Parasitol, 1987. 26(1-2): p. 155-66.

3. Anders, R.F., et al., Immunisation with recombinant AMA-1 protects mice against infection with Plasmodium chabaudi. Vaccine, 1998. 16(2-3): p. 240-7.

4. Crewther, P.E., et al., Protective immune responses to apical membrane antigen 1 of Plasmodium chabaudi involve recognition of strain-specific epitopes. Infect Immun, 1996. 64(8): p. 3310-7.

5. Peterson, M.G., et al., Integral membrane protein located in the apical complex of Plasmodium falciparum. Mol Cell Biol, 1989. 9(7): p. 3151-4.

6. Kocken, C.H., et al., Molecular characterisation of plasmodium reichenowi apical membrane antigen-1 (AMA-1), comparison with P. falciparum AMA-1, and antibody-mediated inhibition of red cell invasion [In Process Citation]. Mol Biochem Parasitol, 2000. 109(2): p. 147-56.

7. Hodder, A.N., et al., The disulfide bond structure of Plasmodium apical membrane antigen-1. J Biol Chem, 1996. 271(46): p. 29446-52.

8. Marshall, V.M., et al., Diversity of the vaccine candidate AMA-1 of Plasmodium falciparum. Mol Biochem Parasitol, 1996. 77(1): p. 109-13.

9. Oliveira, D.A., et al., Genetic conservation of the Plasmodium falciparum apical membrane antigen-1 (AMA-1). Mol Biochem Parasitol, 1996. 76(1-2): p. 333-6.

10. Thomas, A.W., A.P. Waters, and D. Carr, Analysis of variation in PF83, an erythrocytic merozoite vaccine candidate antigen of Plasmodium falciparum. Mol Biochem Parasitol, 1990. 42(2): p. 285-7.

11. Deans, J.A., et al., Vaccination trials in rhesus monkeys with a minor, invariant, Plasmodium knowlesi 66 kD merozoite antigen. Parasite Immunol, 1988. 10(5): p. 535-52.

12. Deans, J.A., et al., Rat monoclonal antibodies which inhibit the in vitro multiplication of Plasmodium knowlesi. Clin Exp Immunol, 1982. 49(2): p. 297-309.

13. Thomas, A.W., et al., The Fab fragments of monoclonal IgG to a merozoite surface antigen inhibit Plasmodium knowlesi invasion of erythrocytes. Mol Biochem Parasitol, 1984. 13(2): p. 187-99.

14. Kocken, C.H., et al., Precise timing of expression of a Plasmodium falciparum-derived transgene in Plasmodium berghei is a critical determinant of subsequent subcellular localization. J Biol Chem, 1998. 273(24): p. 15119-24.

15. Narum, D.L., G.W. Welling, and A.W. Thomas, Ion-exchange-immunoaffinity purification of a recombinant baculovirus Plasmodium falciparum apical membrane antigen, PF83/AMA-1. J Chromatogr A, 1993. 657(2): p. 357-63.

16. Kocken, C.H., et al., High-level expression of Plasmodium vivax apical membrane antigen 1 (AMA-1) in Pichia pastoris: strong immunogenicity in Macaca mulatta immunized with P. vivax AMA-1 and adjuvant SBAS2. Infect Immun, 1999. 67(1): p. 43-9.

17. Garrity, R.R., et al., Refocusing neutralizing antibody response by targeted dampening of an immunodominant epitope. J Immunol, 1997. 159(1): p. 279-89.

18. Withers-Martinez, C., Carpenter, E.P., Hackett, F., Ely, B., Sajid, M., Grainger, M. and Blackman, M.J. (1999) PCR-based gene synthesis as an efficient approach for expression of the A + T-rich malaria genome. Prot. Engineering 12, 1113-1120.

19. Urist, M.R., et al., Purification of bovine bone morphogenetic protein by hydroxyapatite chromatography. Proc Natl Acad Sci U S A, 1984. 81(2): p. 371-

5.

20. Roelcke, D. and H. Jungfer, Subfractionation of gammaG-myeloma globulins by hydroxy-apatite column chromatography. Ger Med Mon, 1971. 1(1): p. 7-8.

CLAIMS

1. A method for producing mRNA encoding *Plasmodium* AMA-1 ectodomain, or a functional part, derivative and/or analogue thereof, in a yeast cell, comprising providing said yeast cell with a nucleic acid encoding said ectodomain or functional part, derivative and/or analogue thereof, said nucleic acid being modified to utilize said yeast's codon usage.

2. A method according to claim 1, further comprising allowing for expression of said ectodomain or functional part, derivative and/or analogue thereof in said yeast cell.

3. A method according to claim 2, further comprising purifying said ectodomain or functional part, derivative and/or analogue thereof.

4. A method according to any one of claims 1-3, wherein at least one putative yeast polyadenylation consensus sequence in said nucleic acid has been modified.

5. A method according to any one of claims 1-4, wherein at least one site in said ectodomain or functional part, derivative and/or analogue thereof that is generally glycosylated by eukaryotic expression systems, has been removed.

6. A method according to any one of claims 1-5, wherein said *Plasmodium* belongs to the clade whose members express said AMA-1 protein as an approximately 83 kDa protein.

7. A method according to any one of claims 1-6, wherein said *Plasmodium* comprises *Plasmodium falciparum*.

8. A method according to any one of claims 1-7, wherein said *Plasmodium* comprises *Plasmodium falciparum* FVO.

9. A method according to any one of claims 1-8, wherein said yeast is *Pichia*.

*10.*A method according to any one of claims 1-9, wherein said yeast is *Pichia pastoris*.

11.An isolated and/or recombinant nucleic acid sequence encoding *Plasmodium*

AMA-1 ectodomain or a functional part, derivative and/or analogue thereof, said nucleic acid being modified to utilize a yeast's codon usage.

12.An isolated and/or recombinant nucleic acid sequence according to claim 11, wherein at least one putative yeast polyadenylation consensus sequence has been modified.

13.An isolated and/or recombinant nucleic acid sequence according to claim 11 or claim 12, wherein at least one site in said ectodomain or functional part, derivative and/or analogue thereof that is generally glycosylated by eukaryotic expression systems, has been removed.

14.An isolated and/or recombinant nucleic acid sequence encoding *Plasmodium* AMA-1 ectodomain or a functional part, derivative and/or analogue thereof, said nucleic acid comprising a sequence as depicted in figure 1.

15.An AMA-1 specific nucleic acid sequence, capable of hybridising to at least a functional part of a nucleic acid according to any one of claims 11-14.

16.A nucleic acid sequence according to claim 15, wherein said hybridisation is under stringent conditions.

17.An AMA-1 specific nucleic acid sequence, comprising at least 50 percent homology to a nucleic acid sequence according to any one of claims 11-14.

18.An AMA-1 specific nucleic acid sequence, comprising at least 60 percent homology to a nucleic acid sequence according to any one of claims 11-14.

19.An AMA-1 specific nucleic acid sequence, comprising at least 75 percent homology to a nucleic acid sequence according to any one of claims 11-12.

20.An AMA-1 specific nucleic acid sequence, comprising at least 90 percent homology to a nucleic acid sequence according to any one of claims 11-14.

21.A nucleic acid sequence according to any one of claims 11-20, wherein said *Plasmodium* belongs to the clade whose members express said AMA-1 protein as an approximately 83 kDa protein.

22.A nucleic acid sequence according to any one of claims 11-21, wherein said *Plasmodium* comprises *Plasmodium falciparum*.

23.A nucleic acid according to any one of claims 11-22, wherein said

Plasmodium comprises *Plasmodium falciparum* FVO.

24.A nucleic acid sequence according to any one of claims 11-23, wherein said ectodomain or functional part, derivative and/or analogue thereof comprises a consensus *Plasmodium* AMA-1 ectodomain or a functional part, derivative and/or analogue thereof.

25.A nucleic acid sequence according to any one of claims 11-24, wherein said yeast is *Pichia*.

26.A nucleic acid sequence according to any one of claims 11-25, wherein said yeast is *Pichia pastoris*.

27.A method for producing *Plasmodium* AMA-1 ectodomain or a functional part, derivative and/or analogue thereof, comprising:

-providing a yeast cell with a nucleic acid according to any one of claims 11-26 and,

-collecting formed *Plasmodium* AMA-1 ectodomain or functional part, derivative and/or analogue thereof.

28.A method according to claim 27, further comprising purifying said ectodomain or functional part, derivative and/or analogue thereof.

29.A method according to claim 27 or claim 28, wherein said yeast is *Pichia*.

30.A method according to claim 29, wherein said yeast is *Pichia pastoris*.

31.A *Plasmodium* AMA-1 ectodomain or a functional part, derivative and/or analogue thereof, obtainable by a method according to any one of claims 27-30.

32.An isolated cell comprising a nucleic acid according to any one of claims 11-26

33.An isolated cell according to claim 32, further comprising a *Plasmodium* AMA-1 ectodomain or a functional part, derivative and/or analogue thereof.

34.Use of a *Plasmodium* AMA-1 ectodomain or functional part, derivative and/or analogue thereof according to claim 31 for the preparation of a vaccine.

35.Use of a *Plasmodium* AMA-1 ectodomain or functional part, derivative and/or analogue thereof according to claim 31 for the preparation of a vaccine for prevention of malaria.

36.Use according to claim 35, wherein said malaria is caused by *Plasmodium falciparum*.

37.Use of a *Plasmodium* AMA-1 ectodomain or functional part, derivative and/or analogue thereof according to claim 31 for diagnosis.

38.A method for, at least in part, prophylaxis of malaria, comprising administering a vaccine according to claim 34 or 35 to an individual.

39.A method according to claim 38, comprising administering to an individual slow release compositions comprising said vaccine.

40.A method for, at least in part, diagnosis of malaria, comprising collecting a sample from an individual and providing *Plasmodium* AMA-1 ectodomain or functional part, derivative and/or analogue thereof according to claim 31 with at least part of said sample.

Title: Efficient expression of *Plasmodium* apical membrane antigen 1 in yeast cells.

Abstract

The present invention provides a means of efficient expression of *Plasmodium* AMA-1 ectodomain or a functional part, derivative and/or analogue thereof in a eukaryotic expression system. Preferably, said *Plasmodium* AMA-1 ectodomain is Pf AMA-1 ectodomain. This protein is preferably expressed in yeast, more preferably in *Pichia pastoris*. Efficient expression is possible using a method for producing mRNA encoding said *Plasmodium* AMA-1 ectodomain in a yeast cell, comprising providing said yeast cell with a nucleic acid encoding said *Plasmodium* AMA-1 ectodomain, said nucleic acid being modified to utilize said yeast's codon usage. Preferably, at least one putative yeast polyadenylation consensus sequence in said nucleic acid has been modified. More preferably, also at least one site in said protein that is generally glycosylated by eukaryotic expression systems, has been removed.

Fig. 1. Synthetic gene for *P. falciparum* FVO strain AMA-1, employing *P. pastoris* codon preference

```
          10         20         30         40         50
ATGAGGAAGTTGTACTGCGTTTTGTTGTTGTCTGCTTTCGAGTTCACCTA
 M  R  K  L  Y  C  V  L  L  L  S  A  F  E  F  T  Y>

          60         70         80         90        100
CATGATCAACTTCGGTCGTGGTCAGAACTACTGGGAGCATCCTTACCAGA
 M  I  N  F  G  R  G  Q  N  Y  W  E  H  P  Y  Q>

         110        120        130        140        150
AGTCTGACGTCTACCATCCTATCAACGAACATAGGGAGCATCCTAAGGAA
K  S  D  V  Y  H  P  I  N  E  H  R  E  H  P  K  E>

         160        170        180        190        200
TACCAATACCCACTGCATCAAGAGCACACTTACCAGCAGGAAGATTCTGG
 Y  E  Y  P  L  H  Q  E  H  T  Y  Q  Q  E  D  S  G>

         210        220        230        240        250
TGAAGATGAAAACACCTTGCAACACGCTTACCCCATCGATCATGAAGGAG
 E  D  E  N  T  L  Q  H  A  Y  P  I  D  H  E  G>

         260        270        280        290        300
CTGAACCAGCCCCTCAGGAACAAAACTTGTTCTCTTCCATCGAAATCGTG
A  E  P  A  P  Q  E  Q  N  L  F  S  S  I  E  I  V>

         310        320        330        340        350
GAAAGATCCAACTACATGGGTAACCCATGGACTGAGTACATGGCAAAGTA
 E  R  S  N  Y  M  G  N  P  W  T  E  Y  M  A  K  Y>

         360        370        380        390        400
CGACATCGAGGAAGTGCACGGAAGTGGTATCAGGGTTGATCTGGGTGAAG
 D  I  E  E  V  H  G  S  G  I  R  V  D  L  G  E>

         410        420        430        440        450
ATGCCGAAGTCGCTGGTACTCAGTACAGACTCCCTTCTGGTAAGTGCCCT
D  A  E  V  A  G  T  Q  Y  R  L  P  S  G  K  C  P>

         460        470        480        490        500
GTTTTCGGAAAGGGTATCATCATCGAAAACTCTAAGACTACTTTCCTCAA
 V  F  G  K  G  I  I  I  E  N  S  K  T  T  F  L  K>

         510        520        530        540        550
GCCTGTTGCTACTGGTAACCAAGATCTTAAGGACGGAGGTTTCGCTTTCC
 P  V  A  T  G  N  Q  D  L  K  D  G  G  F  A  F>

         560        570        580        590        600
CACCTACTAACCCTCTGATCTCTCCAATGACTTTCAACGGTATGCGTGAC
P  P  T  N  P  L  I  S  P  M  T  L  N  G  M  R  D>

         610        620        630        640        650
TTCTACAAGAACAACGAATACGTCAAGAACTTGGATGAATTGACTTTGTG
 F  Y  K  N  N  E  Y  V  K  N  L  D  E  L  T  L  C>

         660        670        680        690        700
TAGTAGACACGCTGGAAACATGAACCCTGATAACGACAAGAACAGTAACT
 S  R  H  A  G  N  M  N  P  D  N  D  K  N  S  N>
```

Fig. 1, Contd.

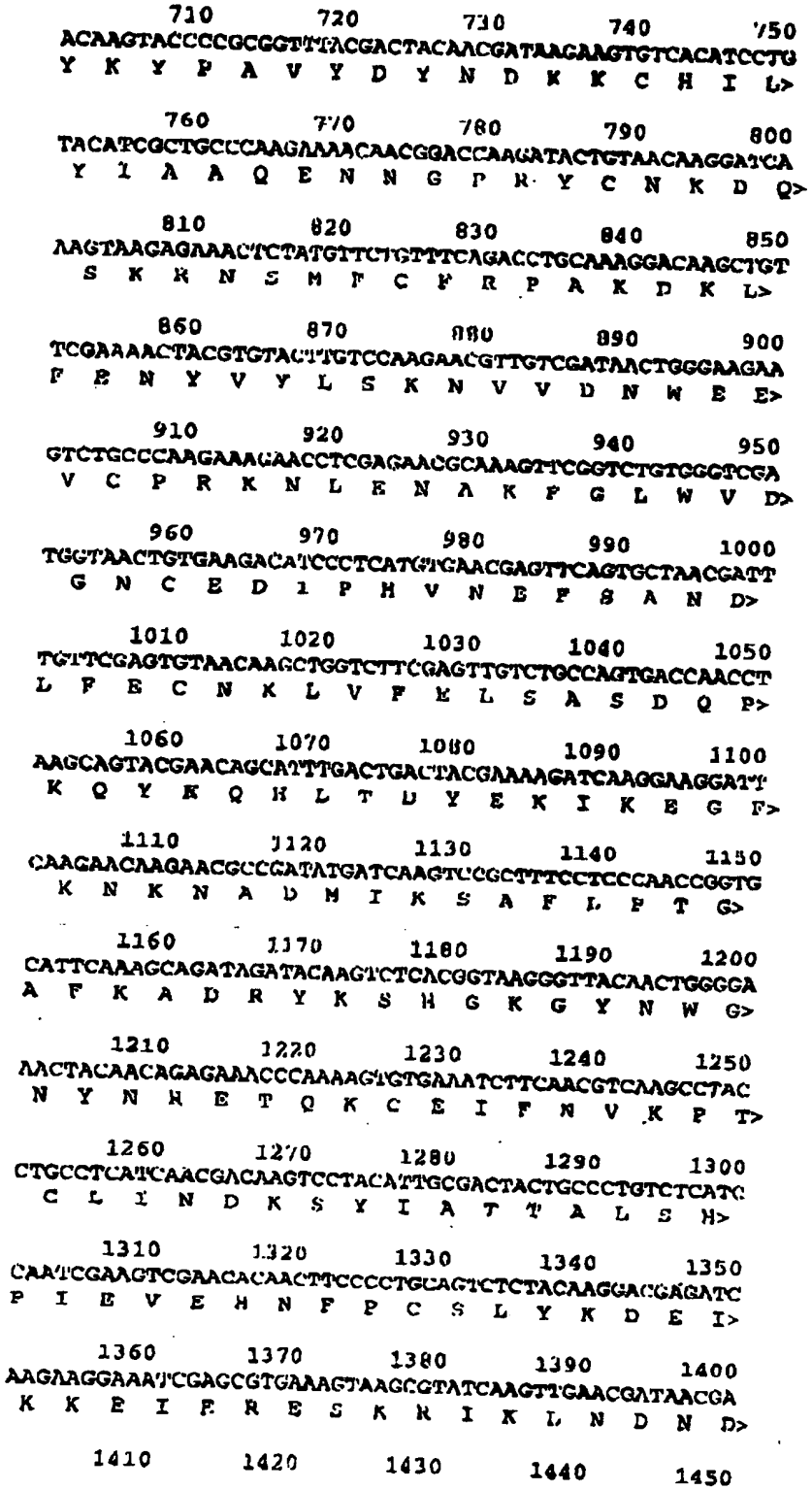
710 720 730 740 750
ACAAGTACCCCGCGGTTTACGACTACAACGATAAGAAGTGTCACATCCTG
Y K Y P A V Y D Y N D K K C H I L>
760 770 780 790 800
TACATCGCTGCCCAAGAAAACAACGGACCAAGATACTGTAACAAGGATCA
Y I A A Q E N N G P R Y C N K D Q>
810 820 830 840 850
AAGTAAGAGAAACTCTATGTTCTGTTTCAGACCTGCAAAGGACAAGCTGT
S K R N S M F C F R P A K D K L>
860 870 880 890 900
TCGAAAACTACGTGTACTTGTCCAAGAACGTTGTCGATAACTGGGAAGAA
F E N Y V Y L S K N V V D N W E E>
910 920 930 940 950
GTCTGCCCAAGAAAGAACCTCGAGAACGCAAAGTTCGGTCTGTGGGTCGA
V C P R K N L E N A K F G L W V D>
960 970 980 990 1000
TGGTAACTGTGAAGACATCCCTCATGTGAACGAGTTCAGTGCTAACGATT
G N C E D I P H V N E F S A N D>
1010 1020 1030 1040 1050
TGTTCGAGTGTAACAAGCTGGTCTTCGAGTTGTCTGCCAGTGACCAACCT
L F E C N K L V F E L S A S D Q P>
1060 1070 1080 1090 1100
AAGCAGTACGAACAGCATTTGACTGACTACGAAAAGATCAAGGAAGGATT
K Q Y E Q H L T D Y E K I K E G F>
1110 1120 1130 1140 1150
CAAGAACAAGAACGCCGATATGATCAAGTCCGCTTTCCTCCCAACCGGTG
K N K N A D M I K S A F L P T G>
1160 1170 1180 1190 1200
CATTCAAAGCAGATAGATACAAGTCTCACGGTAAGGGTTACAACTGGGGA
A F K A D R Y K S H G K G Y N W G>
1210 1220 1230 1240 1250
AACTACAACAGAGAAACCCAAAAGTGTGAAATCTTCAACGTCAAGCCTAC
N Y N R E T Q K C E I F N V K P T>
1260 1270 1280 1290 1300
CTGCCTCATCAACGACAAGTCCTACATTGCGACTACTGCCCTGTCTCATC
C L I N D K S Y I A T T A L S H>
1310 1320 1330 1340 1350
CAATCGAAGTCGAACACAACTTCCCCTGCAGTCTCTACAAGGACGAGATC
P I E V E H N F P C S L Y K D E I>
1360 1370 1380 1390 1400
AAGAAGGAAATCGAGCGTGAAAGTAAGCGTATCAAGTTGAACGATAACGA
K K E I E R E S K R I K L N D N D>
1410 1420 1430 1440 1450

Fig. 1, Contd.

```
CGACGAAGGTAACAAGAAGATCATCCCACCTAGGATCTTCATCTCCGATG
  D  E  G  N  K  K  I  I  A  P  R  I  F  I  S  D>

      1460       1470       1480       1490       1500
ACAAGGATTCCCTCAAGTGTCCTTGTGACCCTGAGATGGTGAGTCAGTCC
D  K  D  S  L  K  C  P  C  D  P  E  M  V  S  Q  S>

      1510       1520       1530       1540       1550
ACTTGTAGATTCTTCGTTGCAAGTGCGTCGAACGTAGAGCCGAAGTCAC
 T  C  R  F  F  V  C  K  C  V  E  R  R  A  E  V  T>

      1560       1570       1580       1590       1600
TAGTAACAACGAAGTTGTCGTGAAGGAAGAATACAAGGATGAATACGCTG
  S  N  N  E  V  V  V  K  E  E  Y  K  D  E  Y  A>

      1610       1620       1630       1640       1650
ATATTCCAGAGCATAAGCCTACGTACGATAACATGAAGATCATCATCGCT
D  I  P  E  H  K  P  T  Y  D  N  M  K  I  I  I  A>

      1660       1670       1680       1690       1700
AGTTCTGCTGCTGTCGCTGTTCTGGCTACTATCCTCATGGTGTACCTTTA
 S  S  A  A  V  A  V  L  A  T  I  L  M  V  Y  L  Y>

      1710       1720       1730       1740       1750
CAAGAGAAAGGGAAACGCTGAGAAGTACGACAAGATGGATCAACCTCAAC
  K  R  K  G  N  A  E  K  Y  D  K  M  D  Q  P  Q>

      1760       1770       1780       1790       1800
ATTACGGTAAGAGTACTTCCAGGAACGATGAGATGTTGGATCCAGAGGCC
H  Y  G  K  S  T  S  R  N  D  E  M  L  D  P  E  A>

      1810       1820       1830       1840       1850
TCCTTCTGGGGTGAGGAGAAGAGAGCCTCTCATACTACTCCAGTTTTGAT
 S  F  W  G  E  E  K  R  A  S  H  T  T  P  V  L  M>

      1860
GGAGAAGCCTTACTACTAA
  E  K  P  Y  Y  *>
```